BACTERIOLOGICAL REVIEWS, Sept., 1973, p. 320-342
Vol. 37, No. 3

Printed in U.S.A.

Proteins from Thermophilic Microorganisms

RIVERS SINGLETON, JR., AND REMI E. AMELUNXEN

Ames Research Center, NASA, Moffett Field, California 94035, and Department of Microbiology, University of Kansas Medical Center, Kansas City, Kansas 66103

INTRODUCTION

Microorganisms are exceptional in their ability to adapt to a wide variety of environmental stresses. One of the more extreme environments is that of elevated temperature. It seems startling to find organisms existing, often obligately, at temperatures which usually preclude the life process, i.e., at temperatures which in vitro can cause the destruction and denaturation of many macromolecules necessary for life.

Because living cells are essentially aqueous chemical systems, their existence is theoretically limited to temperatures at which water exists in a liquid state. However, it is difficult to establish precise temperature limits which are compatible with life. Recent studies have demonstrated the presence of microbial life at temperatures approaching or slightly exceeding the boiling point of water (19, 20). Because most proteins and nucleic acids, as well as many low-molecular-weight compounds, are denatured by temperatures of this nature, any organism existing under these conditions must possess unusual mechanisms which enable its survival.

The mechanisms involved in survival in such an extreme environment are of scientific interest for several reasons. The phenomenon of thermophily would appear to be a primitive characteristic from an evolutionary point of view. Tanaka et al. (127) and Peck (107) have proposed phylogenetic trees of *Clostridium*, indicating that thermophilic species are the oldest from evolutionary considerations. Brock and others (19) have speculated that thermophiles may have retained characteristics of primordial life forms. Thus, a study of the physiology and biochemistry of the thermophilic bacteria will perhaps lead us to a better understanding of how living systems have evolved and developed.

In addition, the possibility of thermal contamination of many aquatic environments be-

comes more significant as the disposal of coolant water from industrial operations into natural environments increases (see Chemical and Engineering News, 12 January 1970, p. 50, for the potential magnitude of this problem). It seems feasible that as the thermal gradient moves downstream from these heat sources, the threat of thermophilic growth in these "thermally polluted" environments may become significant (63). Castenholtz (32) has referred to thermophilic blue-green algal blooms in thermally polluted environments. Brock and Yoder (22) have found thermophilic bacteria growing in the Jordan River (Ind.), which is subject to moderate thermal pollution. Zeikus and Brock (145) have reported an initial study of the Yellowstone Geyser Basin-Firehole River drainage area as a model system of a thermally polluted environment, and they suggested that this system is an excellent model for a long-term study of the ecological consequences of thermal pollution. Although thermophilic microorganisms are generally considered nonpathogenic for man (116), we have little knowledge of what their effects on the ecology of these environments could be.

It should also be pointed out that these organisms and their proteins may be of considerable industrial importance. Historically (2, 55), thermophilic bacteria have been of industrial note for their nuisance value, i.e., as spoilage factors in the canning and related food industries. Their significance in these areas seems to have decreased today. However, as biological systems are increasingly applied to industrial technology, it seems logical that thermophilic bacteria, because of their increased tolerance of heat, will play an important role. Because of their remarkable heat stability, enzymes from thermophiles may be ideally suited for many industrial processes.

Finally, by gaining some insight into the mechanisms of thermophily, we will come to a better understanding of a fundamental life process which, after all, is the ultimate goal of the biological scientist.

It is the thesis of this review that the survival of thermophilic microorganisms is due primarily to an inherent stability of the macromolecular complexes. In considering thermophily, it must be realized that there is probably no single mechanism, but rather a host of molecular variations involved in survival in such an extreme environment. Presently, known physicochemical differences between thermophilic and mesophilic proteins are subtle and give few clues as to a possible molecular mechanism. The intent of this review is to focus specifically on the physicochemical data that have accumulated on thermophilic proteins. Although no unifying concepts are apparent, it is hoped that this information will prove useful in future experimentation dealing with the complexities of thermophily.

Previous reviews in the area of thermophily include those of Gaughran (55), Allen (2), Koffler (72), Brock (19), Campbell and Pace (29), Farrell and Rose (50, 51), and Farrell and Campbell (49). Castenholtz (32) has reviewed the area of thermophilic blue-green algae. Because Friedman (54) has recently reviewed the area of protein synthesis in the thermophiles, we have intentionally not discussed this very important area.

THE THERMOPHILIC MICROORGANISMS

The thermophilic bacteria are a ubiquitous group of microorganisms found in a wide variety of environmental conditions ranging from thermal pools and desert soils to arctic glaciers and freshly fallen snow. As defined by Farrell and Campbell (49), thermophiles can exist at elevated temperatures as obligate, facultative, or thermotolerant organisms. Although the maximum temperature of growth is theoretically limited by the availability of liquid water, Brock (19, 20) has noted that the actual upper limit decreases with increasing acidity of the growth environment. The temperature limits of microbial growth have been considered in some detail by Brock (19).

Thermophilic species are found in most bacterial genera (2, 55). In general appearance they resemble their mesophilic counterparts. They ferment similar carbohydrates, utilize similar nitrogen sources, and have similar oxidative pathways. They can exist as aerobes, anaerobes, or as facultative aerobes; autotrophic and heterotrophic species exist.

Observations of thermophilic growth date from ancient times (19); mechanisms to explain the origin of thermophiles are numerous and often exotic. Arrhenius (8) suggested that thermophilic microbes had their origin on the planet Venus and were carried to the earth by radiation pressure from the sun in a few days. On a more mundane level, arguments regarding their origin have developed along two lines of thought, i.e., either mesophiles developed from thermophiles or vice versa. Allen (2) has presented very persuasive arguments for the mesophilic origin of thermophiles via either adaptation or mutation. The basis of this argument is the ubiquitous occurrence of thermophilic species in nonthermophilic environments and the

finding that some mesophilic species can be adapted to growth at elevated temperatures.

As was previously noted, the evidence available today (127) suggests a thermophilic origin for mesophiles. The most compelling support for this hypothesis comes from the argument that evolution proceeded from an environment considerably warmer than the present one. However, it seems safe to conclude that the origin of thermophilic species is by no means established.

POSSIBLE MECHANISMS OF SURVIVAL

Several general mechanisms have been proposed to explain the survival of thermophilic microorganisms at elevated temperatures. These proposed mechanisms have broadly developed around the following three concepts: (i) stabilization may be achieved through lipid interaction; (ii) heat-denatured cellular components may be rapidly resynthesized; and (iii) thermophilic organisms may possess macromolecular complexes with an inherent heat stability. Each of these possible mechanisms will be considered in turn.

Lipids

Heilbrunn (59) and Bělehrádek (12) observed that thermally stable organisms had lipids with higher melting points than did thermally labile organisms. They suggested that the temperature at which cellular lipids melted might set an upper limit for cellular growth. Other authors (37, 40, 112, 113) have noted that as the growth temperature increases, the percentage of saturated and branched-chain fatty acids increases. These changes could cause a higher melting point and greater flexibility in the membrane lipids. Brock (19) has suggested that changes of this nature might therefore provide the organism with a more stable membrane. A recent study using spin-labeling techniques (34, 35) suggested that membranes prepared from facultative thermophiles grown at 55 C are more rigid than the membranes of cells grown at 37 C. Another study (102) including the same organism demonstrated significant differences in the cell walls of cells grown at 37 and 55 C. Differences were found in chemical composition, susceptibility to lysozyme, and morphological changes observable by electron microscopy. These studies support Brock's hypothesis and suggest that differences do occur in the membranes and cell walls of thermophiles and mesophiles. However, because little is known about the membrane structure of bacteria in general, it is difficult to relate these observations to a generalized mechanism of thermophily. This potentially exciting area must await further developments in the area of membrane biochemistry.

Recently, Wisdom and Welker (137) have reported the observation that the alkaline phosphatase of *Bacillus stearothermophilus* is more thermostable within the cell membrane than in lysed cells. This observation suggests that the cell membrane has the ability to stabilize "soluble" enzymes within the cell. Such a phenomenon could arise from a loose association of the protein with the membrane, some unknown type of architectural arrangement within the cell, or possibly from a simple effect of high protein concentration within the cell. If such observations can be extended to other proteins from thermophiles, they will have important implications for the mechanisms of survival of these organisms.

Rapid Resynthesis

Lamanna (78) has pointed out that in the genus *Bacillus*, smaller organisms tend to have a greater heat resistance than larger species. Copeland (39) made similar observations regarding several genera of blue-green algae. Allen (2) has noted that smaller cells should have a higher metabolic rate due to a greater ratio of surface to volume, because this condition would facilitate the rapid transport of substrates and waste products into and out of the cell.

Based on these and other observations, it has been postulated (2) that growth at elevated temperatures is simply the result of a rapid resynthesis of heat-denatured cellular components. Evidence supporting this hypothesis has been reviewed by Allen (2). (One should note, however, that these studies all involved the use of spore formers, which exhibit a very high rate of protein turnover prior to sporulation.) Further support has been provided by Bubela and Holdsworth (23, 24), who noted that the rates of protein and nucleic acid synthesis and turnover in *B. stearothermophilus* are considerably higher than those of *Escherichia coli*.

However, Koffler (72) has pointed out that if thermophily is simply a kinetic function of rapid resynthesis, then mesophiles should be able to grow at elevated temperatures (and conversely, thermophiles should be able to grow at low temperatures). He suggested that an organism should be anywhere from 16 to 81 times as active at 70 C as it is at 30 C (assuming a doubling or tripling of rate with a 10 C increase in temperature).

If survival at elevated temperatures is related to a simple kinetic function, then it should follow an Arrhenius equation. Brock (19) has compiled the optimal growth temperatures and growth rates for a series of thermophilic and mesophilic organisms. These data were plotted in a double-reciprocal Arrhenius manner. Although the plot was generally linear (with a marked nonlinearity at higher temperatures), the slope was considerably less than that found for a single species extrapolated over the temperature range. The data strongly suggested that the thermophiles do not grow as rapidly at their optima as would be predicted from an Arrhenius relationship and that survival at elevated temperatures is not simply a function of rapid resynthesis of heat-denatured cellular components. It should be noted, however, that the number of data points for this determination were few, and it seems possible that the relationships noted may not be statistically valid.

Thermally Stable Macromolecules

One may consider three possible mechanisms a priori to explain the survival of thermophiles in molecular terms: (i) thermophiles may contain factors which increase the stability of their components with respect to heat; (ii) mesophiles may contain factors which increase the lability of their components with respect to heat; and (iii) cellular components of thermophiles may have an inherent heat stability, independent of exogenous factors.

These hypotheses have been tested (6, 72, 73) to some extent by mixing cell-free extracts from thermophilic microorganisms with extracts prepared from mesophiles and by testing for protein denaturation after heating. The thermophilic extracts were found to possess a marked degree of heat stability, which was not transferable to the mesophilic extract. Similarly, the mesophilic extract caused no loss in thermal stability in the thermophilic extract. It should be noted that these experiments only exclude *transferable* factors from being related to the stabilization of the systems studied. It is conceivable that a stabilizing factor could be so tightly bound as to not be transferable in the experiments described.

In other experiments, Amelunxen and Lins (6) assayed several enzymes from *B. stearothermophilus* and *Bacillus cereus* in crude lysates. Generally, the thermophilic enzymes were considerably more thermostable than the homologous mesophilic enzyme. Howell et al. (66) have reported similar findings with several glycolytic enzymes from thermophilic and mesophilic Clostridia.

Thus far the only example of a thermophilic enzyme being associated with a stabilizing factor (other than a substrate) is a catalase (EC 1.11.1.6) studied in an unspecified thermophile (100). The crude enzyme was observed to possess a high degree of heat stability which decreased upon purification. It was found that a factor (S factor) could be removed from the crude enzyme by charcoal treatment and could be isolated by boiling the crude enzyme. The effect of this factor was slight, however. The temperature optimum for the "free" enzyme was 60 C, whereas the temperature optimum in the presence of S factor was 65 C. Therefore, even in the absence of the factor, the enzyme was heat stable. The "free" enzyme was found to have kinetic behavior similar to other catalases. The physical properties of the enzyme and the nature of S factor have not been reported.

PROTEINS FROM THERMOPHILIC MICROORGANISMS

Most of the available evidence supports the hypothesis that thermophilic microorganisms synthesize macromolecular components which possess an intrinsic thermostability which is independent of any transferable (soluble), stabilizing factors. Although nontransferable stabilizing factors may play some role in the survival of the organism, it will be shown later that such factors must be low molecular weight in nature. In all likelihood, the survival of the organism is the result of the interaction of several mechanisms, e.g., a more stable membrane, more rapid growth, perhaps some type of structural stabilization, and so forth. However, one of the primary contributing factors toward the survival of the organism is the inherent heat stability of its cellular proteins. Without the presence of thermally stable biosynthetic systems, the organisms could not survive.

Currently, approximately 20 proteins have been isolated from a variety of thermophilic microorganisms. Many of these proteins have been highly purified and well characterized physically and chemically. Homologous proteins from mesophilic organisms have been thoroughly described, thus giving a valid basis for comparison with the thermophilic enzymes.

Classification

Farrell and Campbell (49) proposed that enzymes from thermophilic sources be classified into three broad classes. Class I denotes those

enzymes which are stable at the temperature of synthesis (usually 55 to 65 C), but are inactivated at slightly higher temperatures. Class II contains those enzymes which are inactivated at the temperature of synthesis, except when in the presence of substrate. Class III includes those enzymes which are highly heat resistant and are stable at temperatures above the temperature of synthesis.

There are several difficulties with this classification system. First, is the basic problem of assessing thermostability of an enzyme, an area of great confusion in the literature. For instance, many authors assume that an elevated temperature optimum is directly related to thermostability. However, because this parameter is ideally a kinetic value and is determined from initial velocity data, it may not truly reflect thermostability of the protein. It seems to us that the best demonstration of thermostability is the demonstration that the protein survives a fixed temperature for a defined length of time under defined conditions. Because these experiments have not been agreed upon, we suggest that investigators report values for several temperatures and times of incubation. It seems preferable that these data be plotted in semilogarithmic fashion, showing the log of remaining activity versus time, at several values of temperature. Furthermore, we also suggest that investigators obtain samples of appropriate homologous, mesophilic proteins and subject them to identical denaturation experiments for purposes of comparative enzymology.

Second, although the system relates the stability of the enzyme to the growth temperature of the organism, it says nothing about the necessity of the enzyme for the survival of the organism. Thus, an enzyme may maintain full activity after extensive heating at temperatures well above the growth optimum of the organism; however, if its products are not necessary for the survival of the organism, the stability of the enzyme would be irrelevant. This criticism stems directly from a void in the study of thermophilic microorganisms. Little is known regarding the metabolic pathways of these organisms; therefore, it is difficult to ascertain which enzymes are crucial for the survival of the organism.

Finally, this classification scheme omits a potentially very significant class of proteins for thermophilic microorganisms, i.e., it makes no provision for those proteins which are *not stable* under any conditions at the optimal temperature of growth.

Weerkamp and MacElroy (134) have recently reported a study of lactate dehydrogenase (EC 1.1.1.27) from *Bacillus caldolyticus* which would appear to fit this classification. When the enzyme was isolated from cells grown in the absence of brain-heart-infusion media, it was rapidly inactivated by 4 min of heating at 70 C, although the cells were grown at 70 C. Amelunxen and Lins (6) noted that pyruvate kinase (EC 2.7.1.40) and glutamic transaminase (EC 2.6.1.1) in crude lysates of *B. stearothermophilus* were rapidly inactivated by temperatures near the growth maximum for the organism.

These findings have two possible interpretations. The level of enzyme found in the crude extract may be due to a rapid rate of synthesis coupled with a slower rate of inactivation. Thus, when the cells are lysed, a significant amount of enzyme may be present in the extract. However, when this extract is heated, rapid inactivation of the enzyme is observed. Alternatively, it is possible that cellular organization somehow provides stabilization of the protein and that destruction of cell structure causes a loss of protein stability. The true magnitude of this problem is not known, because few studies of this nature are available in the literature. Unfortunately, investigators may not be prone to publish information on nonstable proteins from thermophiles. In reality, this could pose one of the more interesting problems in the area of thermophilic existence.

Red Fraction

One of the first cell-free systems isolated from a thermophilic bacterium was a red-colored particulate fraction which had the ability to oxidize malate, succinate, α-ketoglutarate, pyruvate, and citrate (45, 46, 88, 92, 93). The fraction showed all of the characteristics of a conventional cytochrome system, but had a high degree of heat stability. The system lost only 30% of its activity after 30 min at 65 C and retained 50% of its activity after 90 min. Control systems from mesophilic sources were almost totally inactivated after 1 to 2 min at 65 C. A malate dehydrogenase (EC 1.1.1.37) (88) partially purified from the red fraction was found to have an optimal temperature of 60 C and was stable when heated at 65 C (for an unspecified length of time).

Adenosine Triphosphatase

A primary criticism of the above work was that it involved the use of a particulate fraction which might confer some degree of thermostability to the proteins. To obviate this criticism,

Militzer and his co-workers purified an apyrase (EC 3.6.1.5) (95), an adenosine triphosphatase (ATPase; EC 3.6.1.3) (89, 94), and a pyrophosphatase (EC 3.6.1.1) (PP_iase) (90) from *B. stearothermophilus*. All of these proteins have a high degree of heat stability, especially the ATPase which was stable at 75 C in the presence of Ca^{2+}. The authors suggested that the PP_iase was structurally different from the corresponding yeast enzyme, based on a marked difference in the entropy of activation for the two enzymes. Hachimori et al. (57) have recently obtained the ATPase from *B. stearothermophilus* in homogeneous form and have studied its physical properties. As seen in Table 1, the enzyme is quite heat stable; however, no unusual physical properties have been found for the enzyme when compared with the nonthermostable enzyme referred to in Tables 2 and 4. The authors have suggested that the enzyme undergoes a conformational change at 55 C, based on a discontinuity occurring at this temperature in the Arrhenius plot. The origin of this change is not apparent, but the authors have concluded that it may be related to the presence of a more active species of enzyme at higher temperatures.

Flagella

Koffler and his collaborators (1, 72–75, 84, 138) have studied flagella isolated from thermophilic and mesophilic bacteria and have found that those isolated from thermophiles have a higher degree of heat resistance. Thermostability was measured by following the viscosity changes which occurred upon disruption of the flagellum into its protein component, flagellin. Flagella from *B. stearothermophilus* were stable over a 30-min period at 70 C and were stable to the action of urea and acetamide. Thermophilic flagellin molecules were found to have fewer charged groups upon titration than mesophilic flagellin. Flagella from *E. coli* underwent rapid and pronounced changes in viscosity at 50 C and were severely denatured by urea and acetamide. Koffler speculated that the smaller number of charged groups might cause a decrease in the tendency of the thermophilic flagella to dissociate. It was suggested that additional stabilization might result from more effective hydrogen bonding in the thermophilic flagella.

In more recent work (75, 138), it has been demonstrated that the flagellar filament is constructed of polarly oriented ovoid subunits of flagellin, a unique protein capable of forming normal filaments by self-assembly. Flagellin from *B. stearothermophilus* consists of a single polypeptide chain with a molecular weight of about 50,000; it contains six tyrosine residues and one tryptophan residue. Four of the tyrosine residues were titrated normally, one was titrated with difficulty, and another could not be titrated except in the presence of protein denaturants. One of the tyrosines appeared to be exposed to the medium, because nitration by tetranitromethane did not affect either the capability for self-assembly or thermal stability. Four of the remaining tyrosine residues could be nitrated and affected the properties of the flagellin, depending on the degree of nitration. The behavior of these tyrosine residues together with other evidence had led Koffler to conclude that the thermophilic flagellum is stabilized through the hydrophobic interaction of these tyrosine residues.

Amylase

Campbell (25, 26) reported the purification and crystallization of an α-amylase (EC 3.2.1.1) from *B. coagulans* which retained 90% of its activity after 1 h at 90 C. However, the small yields of the enzyme precluded a detailed study of its properties. Attention was then turned to the α-amylase of *B. stearothermophilus* which could be obtained in higher yields (27, 28, 85, 86). Enzyme biosynthesis was optimally induced at pH 6.7 by maltose and related compounds (135, 136). The enzyme was crystallized and found to be extremely heat stable. There was no loss of enzymatic activity after 24 h of incubation at 65 or 70 C, and only a 29% loss of activity occurred after 20 h at 85 C. This heat stability was also observed under assay conditions.

The properties of the enzyme are summarized in Tables 1 and 3, and demonstrate the protein to be nonspherical and of low molecular weight. A large, negative optical rotation suggested an absence of appreciable helical structure. Furthermore, 8 M urea and 4 M guanidine-HCl had no effect on either the optical rotation or the enzymatic activity. The amino acid composition was of interest from several aspects. First, the protein appeared to be quite acidic, with aspartic and glutamic acids accounting for 23% of the total residues. This composition was reflected in the acidic isoelectric point noted in Table 1. Second, the protein contained 4 mol of half-cystine and no free sulfhydryl groups, suggesting the presence of two disulfide bonds. Performic acid oxidation split the protein into two inactive fragments, further supporting this hypothesis. Third, proline accounted for 15% of the total residues, a value much higher than that found for other α-amylases (by four to five times). Because proline is known to disrupt α-helix

TABLE 1. *Physical properties of proteins from thermophilic microorganisms*[a]

Enzyme	Aldolase			α-Amylase			ATPase	Dehydrogenases				Fdx	FTHFS	G-6-P Isom	Proteolytic enzymes	
				Ref. 88[b]	Ref. 104	Ref. 109		GPDH	MTHFDH	MDH	ICDH				Protease	AP I
Source[c] / Property	5	3	1	1	1	1	1	1	4	1	1	3	4	1	2	1
$s^0_{20,w} \times 10^{-13}$ (s)	—	—	—	0.767	4.2	4.2	11.9	7.17	3.8	6.6	—	—	9.86	7.4	3.5	16.5
$D^0_{20,w} \times 10^{-7}$ (cm^2, s^{-1})	—	—	—	3.85	—	—	—	3.95	—	5.4	—	—	—	—	—	3.9
$\bar{v}$ (mg^{-1})	—	—	—	0.71	—	—	—	0.75	0.752	—	—	0.62	0.752	—	—	—
Mol wt	140,000	68,000	60,600	15,588	48,000	52,700	280,000	148,700	55,000	115,000	92,500	6,000	244,000	172,000	37,500	400,000
f/f_0	—	—	—	1.6	—	—	—	1.38	—	—	—	—	1.27	—	—	1.0
Stokes radius (nm)	—	—	—	—	—	—	—	—	3.17	4.0	—	—	5.3	—	—	5.5
a_0	—	—	+33	—	−225	—	+84	−129	—	—	—	—	—	—	—	—
b_0	—	—	−245	—	−115	—	−147	−134	—	—	—	−23	—	—	81.3	—
Helix (%)	—	—	32.6	—	18.0	—	20.0	—	—	—	—	—	—	—	—	—
pI	—	—	—	4.8	—	9.3	—	4.6		—	—	—	—	—	—	—
Subunits (no.)	2	—	—	1	—	—	—	4	2	—	2	—	4	—	—	12
T_{opt} (C)	95	72	70	70	70	—	65	—	above 64	—	66	55	—	70	68	90
Stability class	III	III	I	III	I	I	III	III	III	I	I	III	III	II	I	III

[a] Abbreviations used: ATPase, adenosine triphosphatase; GPDH, glyceraldehyde-3-phosphate dehydrogenase; MTHFDH, methylene tetrahydrofolate dehydrogenase; ICDH, isocitrate dehydrogenase; Fdx, ferredoxin; FTHFS, formyltetrahydrofolate synthetase; G-6-P Isom, glucose-6-phosphatase isomerase; and AP I, aminopeptidase I.

[b] These data have not been reproduced and may be applicable only to a unique strain of this organism.

[c] Source code: 1, *Bacillus stearothermophilus;* 2, *Bacillus thermoproteolyticus;* 3, *Clostridium tartarivorum;* 4, *Clostridium thermoaceticum;* 5, *Thermus aquaticus.*

TABLE 2. *Physical properties of proteins from representative nonthermophilic sources*[a]

Enzyme	Aldolase	α-Amylase	ATPase	GPDH		MDH		ICDH	Fdx	FTHFS	G-6-P Isom		Protease	AP I
Property \ Source[b]	1	2	3	4	1	2	5	6	7	8	4	1	1	9
$s^0_{20,w}$	5.02	4.4	13.4	7.71	6.80	6.70	4.44	4.6	1.4	9.25	7.19	7.6	3.02	12.56
$D^0_{20,w}$	—	—	—	3.95	5.19	5.57	6.81	5.4	—	3.87	5.15	5.1	8.20	3.75
$\bar{v}$	—	—	0.742	0.754	0.739	—	—	—	0.63	—	0.74	—	0.735	0.751
Mol wt	80,000	49,000	385,000	145,000	122,000	116,800	60,500	80,000	6,012	230,000	132,000	120,000	40,500	326,000
f/f_0	—	—	1.35	1.38	1.25	1.23	1.21	5.0	—	—	1.23	—	1.21	1.23
Stokes radius (nm)	—	—	—	—	—	4.0	3.15	—	—	—	—	—	—	—
a_0	—	−130	—	—	—	—	—	—	—	—	—	—	—	—
b_0	—	−150	—	—	—	—	—	—	—	—	—	—	—	—
Helix (%)	—	25	—	—	—	—	—	30	—	—	—	—	—	—
pI	—	—	—	—	—	—	—	—	—	—	8.5	—	8.5	—
Subunits (no.)	2	—	12	4	4	4	2	1	—	4	—	2	—	10
T_{opt} (C)	—	50	—	—	—	50	45	—	—	42	—	—	—	—
Reference	58	104	119	120	120	98	98	38	128	61	101	101	91	41

[a] See Table 1 for abbreviations of enzyme names.

[b] Source code: 1, *yeast;* 2, *Bacillus subtilis;* 3, *Streptococcus faecalis;* 4, *muscle;* 5, *Escherichia coli;* 6, *Azetobacter vinelandii;* 7, *Clostridium pasteurianum;* 8, *Clostridium cylindosporum;* 9, *bovine lens.*

TABLE 3. *Amino acid composition of proteins from thermophilic microorganisms*[a]

Enzyme	Aldolase	α-Amylase			AP I	ATPase	Fdx	FTHFS	MTHFDH	GPDH	G-6-P Isom	ICDH	Protease
		Ref. 85[b]	Ref. 104	Ref. 109									
Source	1	1	1	1	1	1	3	4	4	1	1	1	2
Asp	51	11	55	61	347	231	4	210	45	160	168	75	43
Thr	28	8	36	37	234	136	4	121	18	80	79	49	23
Ser	26	6	20	30	118	122	1	69	18	72	73	27	23
Glu	67	22	32	35	410	310	7	179	48	112	156	102	20
Pro	21	22	18	16	172	121	2	96	28	44	53	41	8
Gly	62	9	41	47	380	221	5	196	45	104	132	79	36
Ala	62	8	29	35	371	244	10	239	58	164	146	91	28
0.5 Cys	—	4	1	0	0	—	8	24	8	12	—	—	0
Val	49	11	24	29	332	206	4	167	58	164	75	62	24
Met	15	3	8	8	52	60	0	52	8	20	18	18	2
Ile	38	7	16	15	246	198	5	148	40	80	82	65	18
Leu	45	9	30	31	292	203	0	206	43	112	168	52	17
Tyr	11	3	29	25	73	69	1	57	4	32	76	25	29
Phe	17	6	20	21	120	93	0	63	7	20	82	31	10
Lys	40	6	19	28	205	138	2	133	36	96	122	68	12
His	11	4	10	9	84	59	2	32	12	36	35	10	9
Arg	26	3	15	16	179	142	0	97	20	64	62	36	10
Try	—	0	17	22	60	—	0	20	—	12	13	10	5
Amide NH_3	—	3	—	—	275	—	—	100	—	—	—	—	38
Residues/mole	569	145	420	465	3,675	2,553	55	2,117	496	1,384	1,540	831	317
Recovery	100.1	101	—	—	100.8	99.7	100	95	—	97.7	100.1	—	102.3

[a] See Table 1 for abbreviations of enzyme names and source codes.
[b] These data have not been reproduced and may be applicable only to a unique strain of this organism.

formation, this observation supported the previous finding of little or no helical structure.

Based on these observations, Campbell and co-workers proposed that the α-amylase from *B. stearothermophilus* possesses a unique structure which is solely responsible for its extreme thermostability. This structure is that of a semirandom or random-coiled well-hydrated molecule, with any secondary structure arising solely from the presence of disulfide bonds.

However, attempts to reproduce these data by other investigators have been unsuccessful, and the conclusions regarding the unique structure of this enzyme have been questioned by Pfueller and Elliott (109). These authors were unable to obtain amylase activity by the published method (85), but were able to obtain a partially purified preparation by another route. However, this enzyme did not exhibit the properties of the previously described enzyme (27, 28, 86), as can be seen in Tables 1 and 3. The significant differences reside in the molecular weight, isoelectric point, and amino acid analysis, especially with respect to proline, which constitutes only 3.4% of the total residues. Importantly, their preparation did not have the extreme heat stability of the amylase described by Manning and Campbell (85), and it required the presence of Ca^{2+} or a high protein concentration for maximum stabilization. Pfueller and Elliott concluded that " . . . it would seem reasonable to view with some reservation the conclusion that the organism produces an enzyme of unique structure, at least until substantiated by further work" (109).

Thus far, this discrepancy has not been resolved. Farrell and Campbell (49) have stated that Pfueller and Elliott were working with a different strain of *B. stearothermophilus* than that used by Manning and Campbell (85). It is possible that the α-amylase studied by Pfueller and Elliott is the same or at least similar to that studied by Endo (47), who reported the purification of an α-amylase from *B. stearothermophilus* var. *amylolyticus*. The enzyme was stable at 80 C, but lost 50% of its activity at 90 C after 1 h of incubation. The heat stability of the enzyme was a function of Ca^{2+} concentration. Farrell and Campbell (49) have excluded this possibility.

Ogasahara et al. (104) have reported the crystallization of an α-amylase from *B. stearothermophilus* Donk, strain BS-1. An intensive physical characterization of the enzyme demonstrated a close resemblance to the enzyme isolated from *B. subtilis* (Tables 2 and 4). These properties also bear a close similarity to those of the enzyme studied by Pfueller and Elliott (109).

Aldolase

Fructose-1,6-diphosphate aldolase (EC 4.1.2.13) has been purified from a variety of thermophilic bacteria. Thompson and Thompson (130) obtained a nonhomogeneous preparation from *B. stearothermophilus* which was quite heat stable. There was no difference in the enzyme prepared from cells grown at 45 C and that extracted from cells grown at 65 C. Also, the enzyme maintained its thermal stability throughout the purification procedure. These experiments demonstrated that the enzyme is not "heat induced" and that its stabilization is not due to the presence of loosely bound factors which could be removed by purification. On the basis of metal chelator studies, the authors classified the enzyme as a type I aldolase, and therefore similar to aldolases from mammalian sources. However, Freeze and Brock (53) and Sugimoto and Nosoh (124) have presented evidence contrary to this observation.

The aldolase from *B. stearothermophilus* was obtained in homogeneous form by Sugimoto and Nosoh (124) who studied its physical properties, which are summarized in Tables 1 and 3. The enzyme contains two atoms of zinc and is strongly inhibited by ethylenediaminetetraacetate (EDTA). Thus, it most likely is a type II aldolase and resembles the corresponding yeast enzyme. The inhibition by EDTA was very interesting, for it suggested that marked changes in the active site occurred around 45 C. The Arrhenius plot of K_m and V_{max} suggested that the enzyme underwent a change in conformation around 50 to 53 C. Although the nature of the conformational change was not defined, the authors concluded that the thermostability of the enzyme is a result of some factor other than an extraordinary protein sequence of peculiar amino acid composition and protein conformation.

Howard and Becker (65) have recently demonstrated that the aldolase from *B. stearothermophilus* is rapidly inactivated when heated in the presence of its substrates. In the presence of fructose-1,6-diphosphate, the enzyme had only 15% of its initial activity after 10 min at 61 C. In the control experiment, the enzyme only lost 2% of its activity. The authors noted that with inactivation, changes occurred in the molar ellipticity of the enzyme.

Barnes et al. (10) have purified aldolases from two thermophilic (*Clostridium tartarivorum* and *C. thermosaccharolyticum*) and one mesophilic (*C. pasteurianum*) species of *Clostridium* The properties of the aldolase from *C. tartarivorum* are summarized in Table 1. The properties of the aldolase from *C. thermosac-*

TABLE 4. *Amino acid composition of proteins from representative nonthermophilic sources*[a]

Enzyme	Aldolase	α-Amylase	ATPase	GPDH	ICDH	Fdx	FTHFS	G-6-P I	Protease	AP I
Source	1	2	3	4	6	7	8	4	2	9
Asp	79	53	372	152	70	8	100	116	49	257
Thr	37	23	252	88	39	1	49	82	31	134
Ser	39	24	228	76	42	5	30	77	32	157
Glu	78	43	480	72	61	4	74	127	26	289
Pro	33	14	144	48	33	3	29	50	12	150
Gly	60	39	324	128	45	4	79	88	30	230
Ala	67	29	312	128	76	8	93	84	28	288
0.5 Cys	9	—	12	16	3	7	9	12	0	71
Val	45	25	252	136	42	6	65	68	17	177
Met	14	5	84	36	8	0	19	29	2	56
Ile	48	17	228	84	39	5	48	71	13	147
Leu	45	23	348	72	60	0	78	115	23	210
Tyr	24	24	120	36	19	1	11	24	24	49
Phe	29	18	108	56	17	1	23	61	10	106
Lys	51	25	228	104	52	1	73	82	17	187
His	22	12	60	44	13	0	17	47	5	44
Arg	19	17	168	40	24	0	25	43	9	115
Try	9	15	—	12	7	0	3	24	3	36
Amide NH_3	—	—	—	—	—	—	69	108	—	279
Residue/mole	703	406	3,720	1,328	650		825	1,308	331	2,703
Recovery	—	—	—	100	—	100	105	102	—	100
Reference	58	68	119	120	38	128	61	101	91	41

[a] See Table 1 for abbreviations of enzyme names and Table 2 for sources codes.

charolyticum are almost identical to those reported here.

All three enzymes demonstrated similar physical properties. They all appear to be type II aldolases (i.e., they require a metal ion for activity), have very similar molecular weights, and are equally susceptible to denaturants such as urea and guanidine-HCl. The significant difference resides in their thermal stability. The enzyme from *C. pasteurianum* has an optimal temperature of 42 C, whereas the thermophilic enzymes have optima between 70 and 72 C. The mesophilic enzyme is completely inactivated within 5 min at 57 C, whereas the thermophilic enzymes remain stable for up to 4 h under the same conditions.

Freeze and Brock (53) have purified an aldolase from the extreme thermophile, *Thermus aquaticus*, an organism initially isolated from a Yellowstone hot spring and often capable of growth in water approaching the boiling point (21). Its properties are summarized in Table 1. It appears to be a classical type II aldolase, except for an apparent tendency to dimerize. In the presence of cysteine, the molecular weight is reduced to 70,000, thus putting it in the same range as other type II (bacterial) aldolases. The enzyme is extremely thermostable, having a temperature optimum of 95 C. Interestingly, it has little activity at temperatures below 60 C. The authors concluded that except for its extreme heat stability, the aldolase from *T. aquaticus* resembles other class II aldolases in all significant respects. It is interesting that although the amino acyl synthetases of this organism possess a high degree of heat stability (when compared with the homologous system of *E. coli*), they were not as heat stable as the above aldolase (144).

Proteolytic Enzymes

An extracellular protease (EC 3.4.4, not specified) was crystallized from *Bacillus thermoproteolyticus* by Endo (48), who noted that the enzyme was remarkably heat stable. The enzyme has been subjected to extensive physicochemical characterization (91, 105, 106) in an attempt to explain the source of its heat stability. These data demonstrate the enzyme to be a moderately large, typically globular protein, with many properties in common with the enzyme from *B. subtilis*. Nineteen "abnor-

mally titrating" tyrosine groups were found by spectrophotometric titration. However, when the enzyme was first heated at 80 C for 20 min, all of the tyrosine residues could be titrated in a normal manner. Similar observations were made by using fluorescence and optical rotatory techniques. These observations led to the conclusion that the enzyme is stabilized by the presence of tyrosine and/or tryptophan residues existing in a hydrophobic environment. Further stabilization was envisioned as arising from hydrogen bonding of the "abnormally titrating" tyrosine residues buried in the hydrophobic milieu. This structure appears to have many points in common with that proposed for flagellin (75, 138).

Moser et al. (96) and Roncari and Zuber (114, 115) have studied a series of aminopeptidases (EC 3.4.1.2) from strains of *B. stearothermophilus* having different temperature optima for growth. Obligately thermophilic strains produce a great deal of aminopeptidase I (AP I), a thermostable protein, and small amounts of AP II and AP III, which are heat labile. Obligately mesophilic strains produce mostly AP II and AP III and very little AP I. Facultative species produce equal amounts of all three proteins. A similar relationship has been noted for the aminopeptidases of thermophilic fungi (36).

The heat-stable species AP I was isolated in homogeneous form, and its properties were studied. It is extremely thermostable and withstands several hours at 80 C without a loss of activity. Indeed, after 30 min at 80 C, the activity increased by about 20%. The enzyme was found to be a high-molecular-weight globular protein. As shown in Table 1, the presence of 12 subunits suggests a complex structure somewhat similar to that of the bovine lens enzyme (41). The subunit structure of the enzyme has recently been studied (123). Two types of subunits (α, β) were observed to occur in varying proportions in the enzyme, leading to three isoenzyme species ($\alpha_{10}\beta_2$, $\alpha_8\beta_4$, and $\alpha_6\beta_6$). Other isoenzyme species could be formed in vitro. Although the various isoenzymes had different substrate specificities, their thermostabilities were identical. Preliminary electron micrographs suggested that the 12 subunits are assembled in two stacked six-membered rings.

The molecular weight of 400,000 is very close to the values reported for other aminopeptidases from *E. coli* (44), hog kidney (62), and bovine lens (41). It was suggested that the protein had a high degree of hydrophobic character, based on the amino acid analysis. However, no mechanism was apparent to explain the marked thermal stability of the enzyme.

Dehydrogenases

The first intracellular enzyme crystallized from a thermophilic bacterium was glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (3). This enzyme has been well characterized physically and chemically (4, 7, 120).

Except for its marked thermostability, this enzyme bears a striking resemblance to the homologous enzyme isolated from rabbit muscle. Like the muscle enzyme, the thermophilic enzyme contains 4 mol of bound nicotinamide adenine dinucleotide (NAD^+). The coenzyme can be removed from the enzyme without loss of thermostability, and the apoenzyme has been crystallized (5, 126). Like the muscle enzyme, the reaction mechanism of the thermophilic enzyme involves the role of an active sulfhydryl at the catalytic site. In contrast to the muscle enzyme, which contains 13 to 14 ancillary sulfhydryl groups in addition to the four at the active sites, the thermophilic enzyme has no ancillary sulfhydryls. Thus, the remaining four half-cystines (Table 3) must exist as disulfides. There is also a higher level of acidic amino acids in the thermophilic enzyme; therefore, the isoelectric point is considerably more acidic than are the isoelectric points of the corresponding enzymes isolated from other sources. Other points of similarity between the thermophilic and nonthermophilic enzymes include similar molecular weights, an identical number of subunits, similar amino acid composition, and inhibition by reduced NAD^+.

Antibodies have been prepared against the thermophilic enzyme as well as against the yeast and rabbit muscle enzymes (117). The enzymes were inhibited by homologous antisera; however, no cross-reactions were observed. The effect of urea on immunological reactivity suggested that there were marked differences between the thermophilic and yeast enzymes in sensitivity to this denaturant.

Like several of the enzymes from thermophilic sources, the glyceraldehyde-3-phosphate dehydrogenase appears to undergo some type of conformational change around 55 C. When the enzyme is denatured in 8 M urea at various temperatures, a first order denaturation reaction is observed (7). If the rate constants from the denaturation reaction are plotted in an Arrhenius fashion, the plot is linear up to 55 C, at which point it breaks off, thus indicating a rapid increase in activation energy. This behavior suggests some type of conformational change; however, the nature of this change is not understood at this time.

The sequence of the active-site tryptic pep-

tide of glyceraldehyde-3-phosphate dehydrogenase has been determined (18). The peptide (comprising positions 4 to 20 of the prototype sequence) consists of 20 residues in contrast to 17 residues found in most glyceraldehyde-3-phosphate dehydrogenases. The peptide from the thermophilic enzyme differs from the prototype sequence only at position 18, where phenylalanine is unique. In most of the active-site peptides sequenced from this enzyme, position 3 is occupied by a lysine residue; among 14 different examples of the prototype sequence, the only known variation at this site occurs in the lobster enzyme, in which this lysine residue is replaced by threonine. However, in the thermophilic enzyme, position 3 is occupied by histidine; in addition, an adjacent histidine residue is found in position 2. The presence of two adjacent histidine residues is a novel feature of the active-site sequence of the thermophilic enzyme. Whether or not these substitutions are involved in the thermostability of thermophilic glyceraldehyde-3-phosphate dehydrogenase remains to be established.

Bridgen and Harris (17) have recently reported their findings on the amino acid sequence of the monomeric subunit of the enzyme. An overall homology of approximately 70% was found between the thermophilic and yeast enzymes, a value very close to that observed from a comparison of mesophilic glyceraldehyde-3-phosphate dehydrogenases.

Murphy et al. (98) have purified malate dehydrogenase (EC 1.1.1.37) from *B. stearothermophilus*, *B. subtilis*, and *E. coli*. The enzymes extracted from members of the genus *Bacillus* are virtually identical with respect to molecular weights and immunochemical properties, although they both differ from the corresponding enzyme extracted from *E. coli*. The small yields of the thermophilic enzyme prohibited its complete molecular characterization.

An NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42) from *B. stearothermophilus* has been purified by Howard and Becker (64); this enzyme has a temperature optimum of 66 C; however, it requires the presence of substrate for maximal stabilization. The molecular weight of the thermophilic enzyme is slightly higher than that reported for the corresponding enzyme from *Azotobacter vinelandii* (38) and approximately twice that of the mammalian counterpart. The thermophilic enzyme has many physical and kinetic properties similar to those of the bacterial and mammalian enzymes.

Isocitrate dehydrogenase has been purified from the extreme thermophile *T. aquaticus* YT-1 by Ramaley and Hudock (Biochim. Biophys. Acta, 1973, in press). The enzyme was extremely heat stable, exhibiting approximately a 20% loss in activity after 1 h at 80 C. The apparent Arrhenius activation energy of the enzyme was similar to that observed for the enzymes from *B. subtilis* and *Chlamydomonas reinhardi*. A molecular weight of approximately 75,000 to 80,000 was determined by chromatography on Sephadex G-200.

In a recent paper, O'Brien et al. (103) have reported the purification of methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) from *Clostridium thermoaceticum*. The properties of the enzyme are shown in Tables 1 and 3. The molecular weight and apparent K_m values for the thermophilic enzyme do not differ greatly from those of the enzymes from *C. cylindosporum* and *C. formicoaceticum*.

Two bands, each exhibiting enzymatic activity, were always seen upon disk electrophoresis. However, when the purified bands were subjected to electrophoresis a second time, two bands were again seen. This observation suggests an equilibrium relationship for the two forms. Sedimentation equilibrium centrifugation demonstrated a single species of 60,000 molecular weight. Thus, the two electrophoretic species are not products of an association equilibrium, but are probably products of a conformational equilibrium.

The thermostability of the enzyme was significant and was not related to the presence of substrate. However, when the enzyme was heat inactivated, the activity decayed to a constant value, which was dependent upon the temperature of incubation. Such a behavior suggests conversion to a more stable form by heating.

These observations are most interesting and with further study may provide some interesting insights into mechanisms of thermophily.

Ferredoxin

An extensive comparative study has been made of thermophilic and mesophilic ferredoxins in the Clostridia (43, 127). Ferredoxin was isolated from *C. tartarivorum* and *C. thermosaccharolyticum* (thermophilic species) and *C. acidiurici* and *C. pasteurianum* (mesophilic species). Because the properties of the two thermophilic ferredoxins are almost identical, only the values for *C. tartarivorum* ferredoxin are given in Tables 1 and 3. The thermophilic proteins have many properties similar to those of the corresponding proteins extracted from the mesophiles, e.g., molecular weights, absorption spectra, iron and sulfide content, sulfhydryl groups, and total number of

residues. Furthermore, the optical rotatory dispersion spectra were similar for all four proteins and suggested some random coil structure. The only significant physicochemical difference between the ferredoxins was the presence of histidine in the amino acid composition of the thermophilic protein. (It is interesting to note that this amino acid was also found in the active-site peptide of thermophilic glyceraldehyde-3-phosphate dehydrogenase.)

However, despite the close similarities of the thermophilic and mesophilic ferredoxins, the thermophilic proteins are considerably more heat stable. They lost only 5 to 10% of their activity upon heating at 70 C for 1 h, whereas the mesophilic proteins lost 70 to 75% of their activity under the same conditions.

The primary sequence of ferredoxin from *C. tartarivorum* has been determined and compared with sequences of less heat-resistant ferredoxins (127). In ferredoxins from mesophiles, *cys*-8 is always preceded by serine or alanine, but in the thermophilic ferredoxin, *cys*-8 is preceded by glutamic acid. The half-cystine at position 43 in mesophilic ferredoxin is always followed by an alanine; glutamine substitutes for this alanine in thermophilic ferredoxin. Other changes in the primary sequence were noted, but it was felt that the above changes were related to the stabilization of the protein (127). However, the mechanism of this stabilization was not understood. Based on the primary sequence of the ferredoxin, a phylogenetic tree showing the evolutionary relationship of clostridial ferredoxins was proposed (127). Peck (107) has proposed a similar phylogenetic relationship relating the development of plant ferredoxins from bacterial origins. Both studies suggest that the thermophilic species was the oldest of the Clostridia studied.

Formyltetrahydrofolate Synthetase

C. thermoaceticum synthesizes acetate from two moles of carbon dioxide (82). Formyltetrahydrofolate synthetase (EC 6.3.4.3) is believed to function in this pathway (125), and has been purified from *C. thermoaceticum*. The properties of this enzyme were studied in great detail and compared with the homologous enzymes isolated from mesophilic Clostridia (16, 81). Although the enzyme was quite thermostable, its physical properties and amino acid analysis were unremarkable and closely resembled the corresponding mesophilic proteins. A slight difference in parameters of hydrophobicity was noted for the thermophilic protein, but the authors felt this difference was not sufficient to explain the thermostability of the enzyme. The use of these parameters of hydrophobicity will be discussed further in this review.

β-Galactosidase

Ulrich et al. (132) have recently reported the partial purification of β-galactosidase (EC 3.2.1.23) from an extreme thermophile resembling *T. aquaticus*. Lactose, galactose, and melibiose induced the synthesis of the enzymes, whereas glucose had a repressive effect. The enzyme is very heat stable, exhibiting no loss in activity when heated at 80 C for 30 min in the presence of cysteine. The Arrhenius activation energy is 13.7 kcal/mol and is thus comparable to that found for the corresponding enzyme from *E. coli*. The molecular weight was also similar to that of the *E. coli* β-galactosidase. The authors concluded that with the exception of thermostability, there are no major differences between the thermophilic and mesophilic β-galactosidases.

Phosphoglucomutase

Phosphoglucomutase (EC 2.7.5.1) was isolated from *Flavobacterium thermophilum* HB8 by Yoshizaki, Oshima, and Imahori (143). The enzyme is quite heat stable, having a temperature optimum of 75 C. Kinetic studies suggested the catalytic mechanism to be of the "ping pong" type and similar to that of the corresponding enzyme from *E. coli*. Other kinetic properties, such as the requirement for glucose-1,6-diphosphate and magnesium ion, are similar to those of the phosphoglucomutase extracted from *E. coli*. The physical properties of this enzyme have not been reported.

Glucose-6-Phosphate Isomerase

Muramatsu and Nosoh (97) have purified the glucose-6-phosphate isomerase (EC 5.3.1.9) from *B. stearothermophilus*. The preparation was homogeneous in the ultracentrifuge and by gel electrophoresis. The enzyme is stable at 50 C, but is rapidly inactivated at 65 C. Considerable protection was afforded at 65 C by glucose-6-phosphate or 6-phosphogluconate. A plot of K_m versus $1/T$ was linear and presented a sharp discontinuity in slope at 55 C, suggesting a conformational change in the protein at this temperature. However, this change was not detectable by optical rotatory dispersion or circular dichroism, and the nature of the conformational shift was not known. It should be noted that some of the kinetic data in this work may be subject to slight question, as they appear to have been obtained under conditions where the enzyme undergoes heat inactivation.

However, these do not seem sufficient to call into question the basic conclusions of the work.

Enolase

In a recent series of papers Stellwagen, et al. (122) and Barnes and Stellwagen (11) have reported the isolation and characterization of enolase (EC 4.2.1.11) from *T. aquaticus* YT-1 and *Thermus* X-1. The enzyme from *T. aquaticus* had a temperature optimum of 90 C and had the hydrodynamic properties of a globular protein. It consisted of eight apparently identical polypeptide chains of molecular weight of 44,000. No significant changes in the sedimentation coefficient, near-UV absorption spectrum, far UV optical rotatory dispersion, or the apparent K_m's were observed in the temperature range 25 to 85 C.

The enzyme from *Thermus* X-1 was obtained in greater yields and was further characterized. Physical properties observed were: $s^0_{20,w}$ = 13.2*S*, $D^0_{20,w} = 3.42 \times 10^{-7}$ cm^2·sec^{-1}, f/f_0 = 1.30, 382,000 molecular weight.

The amino acid composition for both enzymes was determined. The thermostability of four enolases (rabbit muscle, yeast, *Thermus* X-1, and *T. aquaticus* YT-1) was found to have a positive correlation with the content of residues capable of forming side-chain hydrogen bonds and a negative correlation with the average hydrophobicity.

ALLOSTERIC INTERACTIONS

Brock (19) has speculated that perhaps the thermophilic microorganisms have sacrificed efficiency and control of function to grow at elevated temperatures. This speculation was based on the assumption that induced fit and allosteric interactions between proteins and small molecules required structural flexibility which might be incompatible with a highly cross-linked, rigid, and therefore, heat-stable protein. The possibility of such a phenomenon was specifically studied in the enzymes reported below.

Aspartokinase

Aspartokinase (EC 2.7.2.4) (33, 76, 77) was purified from *B. stearothermophilus* and demonstrated to have a temperature optimum of 55 C. The enzyme catalyzes the first reaction in the aspartate pathway leading to the biosynthesis of lysine, threonine, methionine, and isoleucine and is under feedback control by two of its ultimate products, lysine and threonine. Feedback inhibition decreases with increasing temperature, but is still significant at 55 C. As the assay temperature is increased, the kinetics of inhibition by threonine became increasingly sigmoidal. These results suggest that the enzyme has two or more threonine binding sites whose interaction is strongly temperature dependent. Cooperative kinetics for lysine inhibition could not be shown, and it was therefore concluded that either a single lysine site was present or, if multiple sites were present, they did not interact.

Threonine Deaminase

The first enzyme of the isoleucine biosynthetic pathway, L-threonine deaminase (EC 4.2.1.16), was isolated from *B. stearothermophilus* (129). The enzyme has a temperature optimum of 65 C and is rapidly inactivated at 70 C. This inactivation can be prevented to varying degrees by the addition of pyridoxal phosphate, threonine, or phosphate. The temperature versus initial activity profile of the enzyme was interesting in that a marked increase in enzyme activity occurred at 45 to 50 C, suggesting the possibility of a conformational change occurring at this temperature. The enzyme was subject to feedback inhibition by isoleucine; like aspartokinase, this inhibition decreased with increasing temperatures, but was still significant at 80 C. Kinetic data and molecular weight determinations suggested similarities between the threonine deaminases of *B. stearothermophilus*, *B. subtilis*, and *Salmonella typhimurium*. All had molecular weights of approximately 20,000 and multiple binding sites for isoleucine and threonine.

Higa and Ramaley (60) have reported a study of the threonine deaminase from an extreme thermophile, *Thermus* X-1. The enzyme was quite heat stable with a temperature optimum of 85 to 90 C. However, the enzyme was not subject to strong feedback inhibition by isoleucine, and it was suggested that regulation of isoleucine biosynthesis in this organism may resemble that reported in *Rhodospirillium rubrum*.

Phosphofructokinase

Phosphofructokinase (EC 2.7.1.11) (140, 142) was purified from the extreme thermophile, *F. thermophilum*, and was demonstrated to be an extremely heat-stable enzyme. After 1 h at 90 C, the enzyme lost only 10% of its initial activity. In the presence of phosphoenolpyruvate (PEP), the enzyme exhibited sigmoidal kinetics with respect to fructose-6-phosphate. The PEP inhibition was relieved by adenosine diphosphate (ADP), converting the kinetic pat-

terns to the Michaelis-Menten type. Analogues of ADP (e.g., inosine diphosphate, guanosine diphosphate, cytosine diphosphate, and uridine diphosphate) could not replace ADP. The effects of PEP and ADP were seen both at 30 and 75 C. The authors concluded that the enzyme exhibits allosteric behavior even at extreme temperatures and that the enzyme plays an important role in the regulation of carbohydrate metabolism in the organism.

Fructose-1,6-Diphosphatase

A fructose-1,6-diphosphatase (EC 3.1.3.11) (141) has also been purified from *F. thermophilum*. Catalytic activity and susceptibility to allosteric effectors (inhibition by adenosine monophosphate and activation by PEP) remained unchanged after 1 h at 70 C. Other properties (K_m, cation requirement, pH optimum, substrate inhibition) of the thermophilic enzyme were similar to fructose-1,6-diphosphatase from other sources.

CONCLUSIONS, SPECULATIONS, AND SUMMARY

In response to the question, "How are thermophilic microorganisms capable of survival at temperatures which cause the denaturation of most biological compounds?," we can provide only one conclusion with any certainty: they synthesize compounds which are not readily heat denaturable, or at least they are relatively heat stable in relation to the temperature at which the organism is growing. This answer is not intellectually satisfying, because it says nothing about the molecular mechanism(s) involved. The thermophilic proteins studied thus far seem to bear a most remarkable similarity to their nonthermostable counterparts. Based on the accumulated physicochemical evidence to date concerning thermophilic proteins, the unique and elegant picture proposed for the α-amylase from B. *stearothermophilus* by Campbell et al. (27, 28, 85, 86) appears to be applicable only to that enzyme from a very particular strain of the organism. As such, this enzyme cannot represent a general mechanism of thermophily.

It seems clear from the data discussed thus far that nonprotein stabilizing factors are not *primarily* involved in thermophilic survival. This conclusion can be reached by consideration of four points: (i) soluble factors cannot be demonstrated by transfer experiments; (ii) thermostability is retained even after repeated purification steps and in some cases, recrystallization; (iii) the recoveries of most of the amino acid analyses were essentially quantitative, i.e., all of the weight of the protein can be accounted for by amino acids; and, (iv) the molecular weights of the thermophilic proteins are very close to those of their mesophilic counterparts. Therefore, any stabilizing factors which might be present must be firmly bound and of low molecular weight.

We should note by way of definition that we consider a "factor" to be a unique substance whose primary function in this case would be to provide thermostability for a protein. This definition would exclude common cellular components such as metal ions and substrates. However, this definition may not be entirely justified in light of evidence currently developing regarding the stabilizing effects of metal ions on macromolecules. Levy and Biltonen (79) and Levy et al. (80) have recently demonstrated that the binding of Mg^{2+} to the yeast transfer ribonucleic acid specific for phenylalanine causes marked changes in the thermodynamic properties of the molecule, resulting in an increased thermostability. It is interesting to speculate that perhaps the major difference in proteins from thermophilic and mesophilic sources is the ability to bind certain metal ions more tightly and thereby fold into a more stable conformation.

The suggestion that thermophilic proteins are not under allosteric control also does not appear valid, although this conclusion may be premature because of the rather small number of studies available. However, upon reconsideration, there really is no reason to expect a thermally stable protein to be an inflexible rigid species with a large amount of cross-linking as was suggested (19). Indeed, one pattern which seems to emerge from the amino acid analyses of these proteins is that the capacity for cross-linking via disulfide interactions may be reduced.

Often there appears to be an increased level of hydrophobic amino acids in proteins from thermophilic sources when compared with their counterparts from mesophilic sources. It has been observed by several authors (72, 86, 105) that proteins from thermophilic sources seem somewhat impervious to reagents which were thought to disrupt hydrophobic bonds. Furthermore, Scheraga (118) and Brandts (15) have pointed out that, unlike other bonding interactions, the strength of the hydrophobic interaction increases with temperature, up to around 60 C, after which the energy begins to decrease. Thus, the point of maximum stabilization by hydrophobic interactions should be around 60 C.

Several authors (64, 81, 120) have calculated parameters which purportedly measure the capacity for hydrophobic interaction in a protein from its amino acid composition. These parameters are $H\phi_{ave}$ (14), NPS (133), and p (52). The symbol $H\phi_{ave}$ represents the average free energy change which would occur in transferring the amino acid residues of a protein from a water environment to an organic environment. The NPS function is simply a summation of the nonpolar residues of the protein. The p function is the ratio of the volume of the polar exterior shell of the protein to the volume of its nonpolar interior. As pointed out by Bigelow (14), NPS and $H\phi_{ave}$ are measures of nonpolarity, whereas p is a measure of polarity. Therefore, p should decrease while $H\phi_{ave}$ and NPS should increase for an increase in the hydrophobic nature of a series of proteins.

These values have been calculated (R. Singleton, Jr., 1973, unpublished data) for the proteins discussed above, and for their homologous mesophilic counterparts from published amino acid compositions. The values are summarized in Table 5. It is apparent from this tabulation that although minor differences occur, there is no definite correlation between these parameters of hydrophobicity and the thermal stability of the protein. Goldsach (56) has reported a similar lack of correlation between $H\phi_{ave}$ and thermal stability for a very large number of proteins from a variety of sources.

Although such parameters do not correlate with thermal stability of the protein, hydrophobic interactions may still account for the stabilization of the protein. All of these parameters make the same assumption, i.e., on the "average," the protein will maintain all polar residues on the exterior and all nonpolar residues on the interior of the molecule. Klotz (71) has analyzed several proteins for which three-dimensional structures are known and has noted that many nonpolar residues are on the exterior of the protein and accessible to the solvent. Thus, the basic assumption of these parameters may not be valid. Furthermore, these functions cannot account for pockets or clusters of hydrophobic interaction at or near the enzymatic active site. Thus, the bulk of the protein may undergo some form of denaturation or change in structure, whereas the region forming the active site remains stable. Such a situation was noted for aspartokinase and threonine deaminase from *B. stearothermophilus*, where allosteric interactions were markedly reduced with increasing temperature, without affecting catalytic activity (77). Also, many of the proteins discussed above were noted to undergo some sort of conformational change in the region around 45 to 55 C, without a loss of enzymatic activity.

Stabilization by hydrophobic clusters might be further increased by hydrogen bonds or salt linkages buried within the hydrophobic cluster itself. Thus, these linkages would be insulated from the weakening effects of the polar water environment. Such a hypothesis becomes more attractive when considered from the evolutionary point of view. For example, a thermophile could have its capacity to exist at elevated temperatures reduced by the mutation of two or three very critical "reinforcing" residues in the hydrophobic cluster. According to the observations of Margoliash (87) on cytochrome *c*, the hydrophobic cluster must remain constant over long evolutionary periods.

The one protein in which the complete sequence is known for both thermophilic and mesophilic species is clostridial ferredoxin (127). The changes in sequence observed often involve the exchange of a charged amino acid for a neutral species; the total number of charged amino acids is somewhat higher for the thermophilic protein. In accord with the previously mentioned observations of Margoliash, there are certain hydrophobic regions which appear to be invariant. Thus, one can envision that some of these "extra" charged amino acids in the thermophilic ferredoxin could be buried within the hydrophobic pocket in a salt linkage. Because the phylogenetic tree for *Clostridium* sp. suggests that the thermophilic species is the oldest from an evolutionary point of view, the mesophilic species could have evolved quite readily through a series of minor mutations in which these charged amino acids were lost. These mutations would decrease the thermal stability of the protein without causing sufficient major changes in the protein sequence to disrupt seriously the tertiary and quaternary structure of the protein, and thereby cause a loss in catalytic activity.

As has been noted earlier, several proteins from thermophilic sources have been observed to undergo some type of conformational change around 55 C (7, 9, 57, 97, 124, 129) even though they retain enzymatic activity beyond this temperature. Because this temperature is in the region of maximum stabilization of hydrophobic bonds, this observation might suggest that the conformational change arises from a "melting" of the hydrophobic cluster, without causing a gross change in the active site region. This process could be similar to the situation

TABLE 5. *Value of hydrophobicity parameters for various enzymes from thermophilic and mesophilic sources*

Enzyme	Source	NPS[a]	$H\phi^{a}_{ave}$	p^{a}	Ref. no.
Aldolase	*Bacillus stearothermophilus*	0.32	1.04	0.95	124
	Yeast	0.33	1.09	1.04	58
	Rabbit C	0.35	1.10	0.93	108
	Rabbit A	0.33	1.10	1.07	108
α-Amylase	*B. stearothermophilus*	0.41	1.21	0.84	28
	B. stearothermophilus	0.34	1.09	1.11	109
	B. subtilis	0.33	1.07	1.26	68
	B. marcerans	0.33	1.03	1.03	42
	Aspergillus oryzae	0.36	1.07	1.26	121
	Rabbit pancreatic	0.33	1.04	1.07	83
	Rabbit parotid	0.34	1.05	1.05	83
	Rat pancreatic	0.33	1.05	1.00	83
Adenosine triphosphatase	*B. stearothermophilus*	0.35	1.10	0.97	57
	Streptococcus faecalis	0.32	1.04	1.13	119
	Rabbit sarcoplasmic reticulum	0.35	1.12	0.86	70
Ferredoxin	*Clostridium tartarivorum*	0.22	0.91	0.76	127
	C. pasteurianum	0.29	0.99	0.59	128
	C. acidi-urici	0.31	1.04	0.59	111
	C. buturicum	0.27	0.90	0.58	13
	Micrococcus aerogenes	0.31	1.07	0.63	131
	Desulfovibrio gigas	0.31	1.01	0.78	69
	Chromatium	0.28	0194	1.23	69
	Scenedesimus	0.27	0.94	1.16	69
	Spinach	0.31	0.98	1.14	69
Glyceraldehyde-3-phosphate dehydrogenase	*B. stearothermophilus*	0.34	1.06	0.97	120
	Human red blood cells	0.32	1.05	1.06	69
	Bovine liver	0.32	1.07	0.95	69
	Rabbit muscle	0.33	1.06	0.81	120
	Lobster muscle	0.34	1.10	0.89	120
	Leaf-cutting bee	0.36	1.14	0.90	30
	Flesh fly	0.34	1.10	0.90	30
	Honeybee	0.34	1.13	0.94	30
	Screw worm fly	0.35	1.10	0.87	30
	Bumblebee	0.36	1.16	0.88	30
Isocitrate dehydrogenase	*B. stearothermophilus*	0.34	1.14	0.97	64
	Azotobacter vinelandii	0.33	1.11	1.04	38
	Equine liver	0.31	1.09	1.19	38
Glucose-6-phosphate isomerase	*B. stearothermophilus*	0.37	1.16	1.08	97
	Rabbit muscle	0.34	1.11	0.99	110
	Human muscle	0.35	1.14	0.98	31
Formyltetrahydrofolate synthetase	*C. Thermoaceticum*	0.36	1.16	0.82	81
	C. aceticum	0.34	1.12	0.82	81
	C. cylindrosporum	0.31	1.05	0.93	81
Aminopeptidase	*B. stearothermophilus*	0.36	1.10	0.86	114
	Aeromonas proteolytica	0.30	0.98	1.17	70
	Porcine kidney	0.38	1.18	1.00	70
Protease	*B. thermoproteolyticus*	0.35	1.07	1.30	106
	B. subtilis	0.31	0.99	1.44	139
	A. oryzae	0.29	0.94	1.08	99
	Myxobacter	0.26	0.86	1.38	67

[a] See text for definition of terms.

suggested above, whereby thermal energy is absorbed into "melting" the hydrophobic cluster, without causing marked conformational changes at the active site. If this hydrophobic interaction is related to the thermal stability of the protein, then one should observe a very marked increase in the *rate* of thermal denaturation as the temperature is increased, with a marked transition occurring around 55 to 60 C.

The aldolase from *T. aquaticus* is most interesting when considered in conjunction with the above discussion. As noted earlier, the enzyme

was quite inactive below 58 C, after which its activity increased markedly up to 95 C. This behavior might be the result of a requirement for a "melting" of a crucial hydrophobic cluster, which somehow blocks the active site. As this hydrophobic cluster "melts," active enzyme is formed.

In closing, it should be noted that we have ignored an interesting aspect of the growth of the thermophilic bacteria, i.e., why do many thermophiles exist obligately? Little attention has been paid to why many thermophiles fail to grow at low temperatures. It seems probable that this failure is a reflection of decreased rates of synthesis; however, many of the enzymes purified from thermophiles are quite active at mesophilic growth temperatures (a very marked exception to this is the aldolase from *T. aquaticus*). Perhaps future studies will provide more insight into this very interesting problem.

In conclusion, it appears that thermophilic microorganisms synthesize thermostable proteins and, except for some rather minor points, these proteins appear to be physicochemically similar to their mesophilic counterparts. Their points of similarity include: (i) molecular weights, (ii) subunit composition, (iii) allosteric effectors, (iv) amino acid composition, and (v) primary sequences. Based on present knowledge, we must conclude that the mechanism of thermophily is far more subtle than previously anticipated and that the final answer may come only through a more complete understanding of the three-dimensional structure of the proteins involved. Data from X-ray crystallographic studies, in combination with primary sequence analysis, may furnish greater insight into the solution of this unique biological problem.

ACKNOWLEDGMENTS

We thank the many members of our respective institutions for their comments and suggestions for this manuscript. We especially thank R. D. MacElroy and L. I. Hochstein of Ames Research Center for their lively discussion and helpful criticism.

R. S. is the recipient of a research associateship from the National Academy of Sciences/National Research Council.

LITERATURE CITED

1. Adye, J., H. Koffler, and G. E. Mallett. 1957. The relative thermostability of flagella from thermophilic bacteria. Arch. Biochem. **67**:251–253.
2. Allen, M. B. 1953. The thermophilic aerobic sporeforming bacteria. Bacteriol. Rev. **17**:125–173.
3. Amelunxen, R. E. 1966. Crystallization of thermostable glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus*. Biochim. Biophys. Acta **122**:175–181.
4. Amelunxen, R. E. 1967. Some chemical and physical properties of thermostable glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus*. Biochim. Biophys. Acta **139**:24–32.
5. Amelunxen, R. E., and J. Clark. 1970. Crystallization of thermostable glyceraldehyde-3-phosphate dehydrogenase after removal of coenzyme. Biochim. Biophys. Acta **221**:650–652.
6. Amelunxen, R. E., and M. Lins. 1968. Comparative thermostability of enzymes from *Bacillus stearothermophilus* and *Bacillus cereus*. Arch. Biochem. Biophys. **125**:765–769.
7. Amelunxen, R. E., M. Noelken, and R. Singleton, Jr. 1970. Studies on the subunit structure of thermostable glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus*. Arch. Biochem. Biophys. **141**:447–455.
8. Arrhenius, S. 1927. Die thermophilen Bakterien unde der Strahlungsdruck der Sonne. Z. Phys. Chem. (Leipzig) **130**:516–519.
9. Babel, W., H. A. Rosenthal, and S. Rapoport. 1972. A unified hypothesis on the causes of the cardinal temperatures of microorganisms; the temperature minimum of *Bacillus stearothermophilus*. Acta Biol. Med. Germ. **28**:565–576.
10. Barnes, E. M., J. M. Akagi, and R. H. Himes. 1971. Properties of fructose-1, 6-diphosphate aldolase from two thermophilic and a mesophilic *Clostridia*. Biochim. Biophys. Acta **227**:199–203.
11. Barnes, L. D., and E Stellwagen. 1973. Enolase from the thermophile *Thermus* x-1. Biochemistry **12**:1559–1565.
12. Bělehrádek S. 1931. Le mecanisme physicochimique de l'adaptation thermique. Protoplasma **12**:406–434.
13. Benson, A. M., H. F. Mower, and K. T. Yasunobu. 1967. The amino acid sequence of *Clostridium butyricum* ferredoxin. Arch. Biochem. Biophys. **121**:563–575.
14. Bigelow, C. C. 1967. On the average hydrophobicity of proteins and the relation between it and protein structure. J. Theor. Biol. **16**:187–211.
15. Brandts, J. F. 1967. Heat effects on proteins and enzymes, p. 25–72. *In* A. H. Rose (ed.), Thermobiology. Academic Press Inc., New York.
16. Brewer, J. M., L. Ljungdahl, T. E. Spencer, and S. H. Neece. 1970. Physical properties of formyltetrahydrofolate synthetase from *Clostridium thermoaceticum*. J. Biol. Chem. **245**:4798–4803.
17. Bridgen, J., and J. I. Harris. 1973. Studies of the primary structure of glycolytic enzymes from *B. stearothermophilus*. Int. Congr. Biochem., 9th, Stockholm, 1–7 July 1973.
18. Bridgen, J., J. I. Harris, P. W. McDonald, R. E. Amelunxen, and J. R. Kimmel. 1972. Amino acid sequence around the catalytic site in glyceraldehyde-3-phosphate dehydrogenase

from *Bacillus stearothermophilus*. J. Bacteriol. **111**:797-800.
19. Brock, T. D. 1967. Life at high temperatures. Science **158**:1012-1019.
20. Brock, T. D., and G. K. Darland. 1970. Limits of microbial existence: temperature and pH. Science **169**:1316-1318.
21. Brock, T. D., and H. Freeze. 1969. *Thermus aquaticus* gen. n. and sp. n., a nonsporulating extreme thermophile. J. Bacteriol. **98**:289-297.
22. Brock, T. D., and I. Yoder. 1970. Thermal pollution of a small river by a large university: bacteriological studies. Proc. Indiana Acad. Sci. **80**:183-188.
23. Bubela, B., and E. S. Holdsworth. 1966. Amino acid uptake, protein and nucleic acid synthesis and turnover in *Bacillus stearothermophilus*. Biochim. Biophys. Acta **123**:364-375.
24. Bubela, B., and E. S. Holdsworth. 1966. Protein synthesis in *Bacillus stearothermophilus*. Biochim. Biophys. Acta **123**:376-389.
25. Campbell, L. L. 1954. Crystallization of α-amylase from a thermophilic bacterium. J. Amer. Chem. Soc. **76**:5256.
26. Campbell, L. L. 1955. Purification and properties of an α-amylase from facultative thermophilic bacteria. Arch. Biochem. Biophys. **54**:154-161.
27. Campbell, L. L., and P. D. Cleveland. 1961. Thermostable α-amylase of *Bacillus stearothermophilus*. IV. amino-terminal and carboxyl-terminal amino acid analysis. J. Biol. Chem. **236**:2966-2969.
28. Campbell, L. L., and G. B. Manning. 1961. Thermostable α-amylase of *Bacillus stearothermophilus*. III. amino acid composition. J. Biol. Chem. **236**:2962-2965.
29. Campbell, L. L., and B. Pace. 1968. Physiology of growth at high temperatures. J. Appl. Bacteriol. **31**:24-35.
30. Carlson, C. W., and R. W. Brosemer. 1971. Comparative structural properties of insect triose phosphate dehydrogenase. Biochemistry **10**:2113-2119.
31. Carter, N. D., and A. Yoshida. 1969. Purification and characterization of human phosphoglucose isomerase. Biochim. Biophys. Acta **181**:12-19.
32. Castenholz, R. W. 1969. Thermophilic bluegreen algae and the thermal environment. Bacteriol. Rev. **33**:476-504.
33. Cavari, B. Z., H. Arkin-Shlank, and N. Grosswicz. 1972. Regulation of aspartokinase activity in a thermophilic bacterium. Biochim. Biophys. Acta **261**:161-167.
34. Chan, M., Y. P. Virmani, R. H. Himes, and J. M. Akagi. 1972. Spin-labeling studies on the molecular environment in the cell membrane of a facultative thermophilic bacterium. Annu. Meet. Amer. Soc. Microbiol., 72nd, Philadelphia, 1972, p. 177.
35. Chan, M., Y. P. Virmani, R. H. Himes, and J. M. Akagi. 1972. Spin-labeling studies on the cell membrane of a facultative thermophilic bacterium. Conf. Extreme Environ., Ames Research Center, NASA, Moffett Field, Calif., 26-28 June 1972.
36. Chapais, R., and H. Zuber. 1970. Thermophilic aminopeptidases: AP I from *Talaromyces duponti*, p. 552-555. *In* G. E. Perlmann and L. Lorand (ed.), Methods in enzymology, vol. 19. Academic Press Inc., New York.
37. Cho. K. Y., and M. R. J. Salton. 1966. Fatty acid composition of bacterial membrane and wall lipids. Biochim. Biophys. Acta **116**:73-79.
38. Chung, A. E., and J. S. Franzen. 1969. Oxidized triphosphopyridine nucleotide specific isocitrate dehydrogenase from *Azotobacter vinelandii* isolation and characterization. Biochemistry **8**:3175-3183.
39. Copeland, J. J. 1936. Yellowstone thermal myxophyceae. Ann. N. Y. Acad. Sci. **36**:1-229.
40. Daron, H. H. 1970. Fatty acid composition of lipid extracts of a thermophilic *Bacillus* species. J. Bacteriol. **101**:145-151.
41. Delange, R. J., and E. L. Smith. 1971. Leucine aminopeptidase and other N-terminal exopeptidases, p. 81-118. *In* P. D. Boyer (ed.), The enzymes, vol. 3, 3rd ed. Academic Press Inc., New York.
42. De Pinto, J. A., and L. L. Campbell. 1968. Purification and properties of the amylase of *Bacillus macerans*. Biochemistry **7**:114-120.
43. Devanathan, T., J. M. Akagi, R. T. Hersh, and R. H. Himes. 1969. Ferredoxin from two thermophilic *Clostridia*. J. Biol. Chem. **244**:2846-2853.
44. Dick, A. J., A. T. Matheson, and J. H. Wang. 1970. A ribosomal-bound aminopeptidase in *Escherichia coli* B: purification and properties. Can. J. Biochem. **48**:1181-1188.
45. Downey, R. J. 1961. Ph.D. thesis, Univ. of Nebraska, Lincoln. Diss. Abstr. **22**:2548.
46. Downey, R. J., C. E. Georgi, and W. E. Militzer. 1962. Electron transport particles from *Bacillus stearothermophilus*. J. Bacteriol. **83**:1140-1146.
47. Endo, S. 1959. Amylase produced by thermophilic bacteria. Hakko Kogaku Zasshi **37**:353-410. (Chem. Abstr. **55**:2790 [1961].)
48. Endo, S. 1962. Studies on protease produced by thermophilic bacteria. Hakko Kogaku Zasshi **40**:346-353. (Chem. Abstr. **62**:5504 [1965].)
49. Farrell, J., and L. L. Campbell. 1969. Thermophilic bacteria and bacteriophages. Advan. Microbial Physiol. **3**:83-109.
50. Farrell, J., and A. Rose. 1967. Temperature effects on microorganisms. Annu. Rev. Microbiol. **21**:101-120.
51. Farrell, J., and A. H. Rose. 1967. Temperature effects on microorganisms, p. 147-218. *In* A. H. Rose (ed.), Thermobiology. Academic Press Inc., New York.
52. Fisher, H. F. 1964. A limiting law relating the size and shape of protein molecules to their composition. Proc. Nat. Acad. Sci. U.S.A. **51**:1285-1291.
53. Freeze, H., and T. D. Brock. 1970. Thermostable aldolase from *Thermus aquaticus*. J. Bacte-

riol. **101**:541-550.
54. Friedman, S. M. 1968. Protein-synthesizing machinery of thermophilic bacteria. Bacteriol. Rev. **32**:27-38.
55. Gaughran, E. R. L. 1947. The thermophilic microorganisms. Bacteriol. Rev. **11**:189-225.
56. Goldsack, D. E. 1970. Relation of the hydrophobicity index to the thermal stability of homologous proteins. Biopolymers **9**:247-252.
57. Hachimori, A., N. Muramatsu, and Y. Nosoh. 1970. Studies on an ATPase of thermophilic bacteria. I. purification and properties. Biochim. Biophys. Acta **206**:426-437.
58. Harris, C. E., R. D. Kobes, D. C. Teller, and W. J. Rutter. 1969. The molecular characteristics of yeast aldolase. Biochemistry **8**:2442-2454.
59. Heilbrunn, L. V. 1924. The colloid chemistry of protoplasm. IV. the heat coagulation of protoplasm. Amer. J. Physiol. **69**:190-199.
60. Higa, E. H., and R. F. Ramaley. 1973. Purification and properties of threonine deaminase from the X-1 isolate of the genus *Thermus*. J. Bacteriol. **114**:556-562.
61. Himes, R. W., and J. C. Rabinowitz. 1962. Formyltetrahydrofolate synthetase. II. Characteristics of the enzyme and the enzymic reaction. J. Biol. Chem. **237**:2903-2914.
62. Himmelhoch, S. R., and E. A. Peterson. 1968. Preparation of leucine aminopeptidase free of endopeptidase activity. Biochemistry **7**:2085-2093.
63. Horvath, R. S., and M. M. Brent. 1972. Thermal pollution and the aquatic microbial community: possible consequences. Environ. Pollut. **3**:143-146.
64. Howard, R. L., and R. R. Becker. 1970. Isolation and some properties of the triphosphopyridine nucleotide isocitrate dehydrogenase from *Bacillus stearothermophilus*. J. Biol. Chem. **245**:3186-3194.
65. Howard, R. L., and R R. Becker. 1972. Substrate inactivation of fructose-1, 6-diphosphate aldolase from *Bacillus stearothermophilus*. Biochim. Biophys. Acta **268**:249-252.
66. Howell, N., J. M. Akagi, and R. H. Himes. 1969. Thermostability of glycolytic enzymes from thermophilic *Clostridia*. Can. J. Microbiol. **15**:461-464.
67. Jackson, R. L., and G. R. Matsueda. 1970. Myxobacter AL-1 protease, p. 591-599. *In* G. E. Perlman and L. Lorand (ed.), Methods in enzymology, vol. 19. Academic Press Inc., New York.
68. Junge, J. M., E. A. Stein, H. Neurath, and E. H. Fischer. 1959. The amino acid composition of α-amylase from *Bacillus subtilis*. J. Biol. Chem. **234**:556-562.
69. Kirschenbaum, D. M. 1971. A compilation of amino acid analyses of proteins. I. Anal. Biochem. **44**:159-173.
70. Kirschenbaum, D. M. 1972. A compilation of amino acid analyses of proteins. II. Anal. Biochem. **49**:248-266.
71. Klotz, I. M. 1970. Comparison of molecular structures of proteins: helix content; distribution of apolar residues. Arch. Biochem. Biophys. **138**:704-706.
72. Koffler, H. 1957. Protoplasmic differences between mesophiles and thermophiles. Bacteriol. Rev. **21**:227-240.
73. Koffler, H., and G. O. Gale. 1957. The relative thermostability of cytoplasmic proteins from thermophilic bacteria. Arch. Biochem. Biophys. **67**:249-251.
74. Koffler, H., G. E. Mallett, and J. Adye. 1957. Molecular basis of biological stability to high temperature. Proc. Nat. Acad. Sci. U.S.A. **43**:464-477.
75. Koffler, H., and L. R. Yarbrough. 1972. The likely participation of hydrophobic bonds in flagellin-flagellin interactions in thermophile flagella. Conf. Extreme Environ., Ames Research Center, NASA, Moffett Field, Calif., 26-28 June 1972.
76. Kuramitsu, H. K. 1968. Concerted feedback inhibition of aspartokinase from *Bacillus stearothermophilus*. Biochim. Biophys. Acta **167**:643-645.
77. Kuramitsu, H. K. 1970. Concerted feedback inhibition of aspartokinase from *Bacillus stearothermophilus*. I. catalytic and regulatory properties. J. Biol. Chem. **245**:2991-2997.
78. Lamanna, C. 1940. Relation between temperature growth range and size in the genus *Bacillus* J. Bacteriol. **39**:593-596.
79. Levy, J., and R. Biltonen. 1972. Thermodynamic studies of transfer ribonucleic acids. III. Thermodynamic model for the thermal unfolding of yeast phenylalanine-specific transfer ribonucleic acid. Biochemistry **11**:4145-4152.
80. Levy, J., G. Rialdo, and R. Biltonen. 1972. Thermodynamic studies of transfer ribonucleic acids. II. Characterization of the thermal unfolding of yeast phenylalanine-specific transfer ribonucleic acid. Biochemistry **11**:4138-4144.
81. Ljungdahl, L., J. M. Brewer, S. H. Neece, and T. Fairwell. 1970. Purification, stability and composition of formyltetrahydrofolate synthetase from *Clostridium thermoaceticum*. J. Biol. Chem. **245**:4791-4797.
82. Ljungdahl, L. G., and H. G. Wood. 1969. Total synthesis of acetate from CO_2 by heterotrophic bacteria. Annu. Rev. Microbiol. **23**:515-538.
83. Malacinski, G. M., and W. J. Rutter. 1969. Multiple molecular forms of α-amylase from the rabbit. Biochemistry **8**:4382-4390.
84. Mallett, G. E., and H. Koffler. 1957. Hypothesis concerning the relative stability of flagella from thermophilic bacteria. Arch. Biochem. Biophys. **67**:254-256.
85. Manning, G. B., and L. L. Campbell. 1961. Thermostable α-amylase of *Bacillus stearothermophilus*. I. Crystallization and some general properties. J. Biol. Chem. **236**:2952-2957.
86. Manning, G. B., L. L. Campbell, and R. J. Foster. 1961. Thermostable α-amylase of *Bacillus stearothermophilus*. II. physical properties and molecular weight. J. Biol. Chem. **236**:2958-2961.

87. Margoliash, E., and A. Schejter. 1966. Cytochrome C. Advan. Protein Chem. **21**:113-286.
88. Marsh, C., and W Militzer. 1952. Thermal enzymes. V. Properties of a malic dehydrogenase. Arch. Biochem. Biophys. **36**:269-275.
89. Marsh, C., and W. Militzer. 1956. Thermal enzymes. VII. Further data on an adenosine triphosphatase. Arch. Biochem. Biophys. **60**:433-438.
90. Marsh, C., and W. Militzer. 1956. Thermal enzymes. VIII. Properties of a heat stable inorganic pyrophosphatase. Arch. Biochem. Biophys. **60**:439-451.
91. Matsubara, H., and J. Feder. 1971. Other bacterial, mold, and yeast proteases, p. 721-795. *In* P. D. Boyer (ed.), The enzymes, vol. 3, 3rd ed. Academic Press Inc., New York.
92. Militzer, W., T. B. Sonderegger, and C. E. Georgi. 1950. Thermal enzymes. II. Cytochromes. Arch. Biochem. **26**:299-306.
93. Militzer, W., T. B. Sonderegger, L. C. Tuttle, and C. E. Georgi. 1949. Thermal enzymes. Arch. Biochem. **24**:75-82.
94. Militzer, W., and L. C. Tuttle. 1952. Thermal enzymes. IV. Partial separation of an adenosine triphosphatase from an apyrase fraction. Arch. Biochem. Biophys. **39**:379-386.
95. Militzer, W., L. C. Tuttle, and C. E. Georgi. 1951. Thermal enzymes. III. Apyrase from a thermophilic bacterium. Arch. Biochem. Biophys. **31**:416-423.
96. Moser, P., G. Roncari, and H. Zuber. 1970. Thermophilic aminopeptidases from *Bac. stearothermophilus*. II. Aminopeptidase I (AP I): physicochemical properties; thermostability and activation; formation of the apoenzyme and subunits. Int. J. Protein Res. **2**:191-207.
97. Muramatsu, N., and Y. Nosoh. 1971. Purification and characterization of glucose-6-phosphate isomerase from *Bacillus stearothermophilus*. Arch. Biochem. Biophys. **144**:245-252.
98. Murphy, W. H., C. Barnaby, J. F. Lin, and N. O. Kaplan. 1967. Malate dehydrogenase. II. Purification and properties of *Bacillus stearothermophilus* and *Escherichia coli* malate dehydrogenase. J. Biol. Chem. **242**:1548-1559.
99. Nakagawa, Y. 1970. Alkaline proteinases from *Aspergillus*, p. 581-591. *In* G. E. Perlman and L. Lorand (ed.), Methods in enzymology, vol. 19. Academic Press Inc., New York.
100. Nakamura, Y., 1960. Studies on the catalase of a thermophilic bacterium. J. Biochem. (Tokyo) **48**:295-307.
101. Noltman, E. A. 1972. Aldose-ketose isomerases., p. 271-354. *In* P. D. Boyer (ed.), The enzymes, vol. 6, 3rd ed. Academic Press Inc., New York.
102. Novitsky, T. J., M. Chan, R. H. Himes, and J. M. Akagi. 1972. Studies on the cell wall of two facultative thermophilic bacteria. Annu. Meet. Amer. Soc. Microbiol., 72nd, Philadelphia, 1972, p. 65.
103. O'Brien, W. E., J. M. Brewer, and L. G. Ljungdahl. 1973. Purification and characterization of thermostable 5,10-methylenetetrahydrofolate dehydrogenase from *Clostridium thermoaceticum*. J. Biol. Chem. **248**:403-408.
104. Ogasahara, K., A. Imanishi, and T. Isemur. 1970. Studies on thermophilic α-amylase from *Bacillus stearothermophilus*, J. Biochem. (Tokyo) **67**:65-89.
105. Ohta, Y. 1967. Thermostable protease from thermophilic bacteria. II. Studies on the stability of the protease. J. Biol. Chem. **242**:509-515.
106. Ohta, Y., Y. Ogura, and A. Wada. 1966. Thermostable protease from thermophilic bacteria. I. Thermostability, physico-chemical properties, and amino acid composition. J. Biol. Chem. **241**:5919-5925.
107. Peck, H. D., Jr. 1972. Chemoautotrophy: multiple adaptations to extreme environments. Conf. Extreme Environ., Ames Research Center, NASA, Moffett Field, Calif., 26-28 June 1972.
108. Penhoet, E. E., M. Kochman, and W. J. Rutter. 1969. Molecular and catalytic properties of aldolase C. Biochemistry **8**:4396-4402.
109. Pfueller, S. L., and W. H. Elliott. 1969. The extracellular α-amylase of *Bacillus stearothermophilus*. J. Biol. Chem. **244**:48-54.
110. Pon, N. G., K. D. Schnackerz, M. N. Blackburn, G. C. Chatterjee, and E. A. Noltmann. 1970. Molecular weight and amino acid composition of five times crystallized phosphoglucose isomerase from rabbit skeletal muscle. Biochemistry **9**:1506-1514.
111. Rall, S. C., R. E. Bolinger, and R. D. Cole. 1969. The amino acid sequence of ferredoxin from *Clostridium acidi-urici*. Biochemistry **8**:2486-2496.
112. Ray, P. H., D. C. White, and T. D. Brock. 1971. Effect of temperature on the fatty acid composition of *Thermus aquaticus*. J. Bacteriol. **106**:25-30.
113. Ray, P. H., D. C. White, and T. D. Brock. 1971. Effect of growth temperature on the lipid composition of *Thermus aquaticus*. J. Bacteriol. **108**:227-235.
114. Roncari, G., and H. Zuber. 1969. Thermophilic aminopeptidases from *Bacillus stearothermophilus*. I. Isolation, specificity, and general properties of the thermostable aminopeptidase I. Int. J. Protein Res. **1**:45-61.
115. Roncari, G., and H. Zuber. 1970. Thermophilic aminopeptidases: AP I from *Bacillus stearothermophilus*, p. 544-552. *In* G. E. Perlmann and L. Lorand (ed.), Methods in enzymology, vol. 19. Academic Press Inc., New York.
116. Sattar, S. A., E. J. Synek, J. C. N. Westwood, and P. Neals. 1972. Hazard inherent in microbial tracers: reduction of risk by the use of *Bacillus stearothermophilus* spores in aerobiology. Appl. Microbiol. **23**:1053-1059.
117. Sauvan, R. L., O. J. Mira, and R. E. Amelunxen. 1972. Thermostable glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus*. I. Immunochemical studies. Biochim. Biophys. Acta **263**:794-804.
118. Scheraga, H. A. 1963. Intramolecular bonds in

proteins. II. Noncovalent bonds, p. 477-594. *In* H. Neurath (ed.), The proteins, vol. 1, 2nd ed. Academic Press Inc., New York.

119. Schnebli, H. P., A. E. Vatter, and A. Abrams. 1970. Membrane adenosine triphosphatase from *Streptococcus faecalis*: molecular weight, subunit structure, and amino acid composition. J. Biol. Chem. **245**:1122-1127.
120. Singleton, R., Jr., J. R. Kimmel, and R. E. Amelunxen. 1969. The amino acid composition and other properties of thermostable glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus*. J. Biol. Chem. **244**:1623-1630.
121. Stein, E. A., J. M. Junge, and E. H. Fischer. 1960. The amino acid composition of α-amylase from *Aspergillus oryzae*. J. Biol. Chem. **235**:371-378.
122. Stellwagen, E., M. M. Cronlund, and L. D. Barnes. 1973. A thermostable enolase from the extreme thermophile *Thermus aquaticus* YT-1. Biochemistry **12**:1552-1558.
123. Stoll, E., M. A. Hermodson, L. H. Ericsson, and H. Zuber. 1972. Subunit structure of the thermophilic aminopeptidase I from *Bacillus stearothermophilus*. Biochemistry **11**:4731-4735.
124. Sugimoto, S., and Y. Nosoh. 1971. Thermal properties of fructose-1, 6-diphosphate aldolase from thermophilic bacteria. Biochim. Biophys. Acta **235**:210-221.
125. Sun, A. Y., L. Ljungdahl, and H. G. Wood. 1969. Total synthesis of acetate from CO_2. II. Purification and properties of formyltetrahydrofolate synthetase from *Clostridium thermoaceticum*. J. Bacteriol. **98**:842-844.
126. Suzuki, K., and J. I. Harris. 1971. Glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus*. FEBS Lett. **13**:217-220.
127. Tanaka, M., M. Haniu, G. Matsueda, K. Yasunobu, R. H. Himes, J. M. Akagi, E. M. Barnes, and T. Devanathan. 1971. The primary structure of the *Clostridium tartarivorum* ferredoxin, a heat-stable ferredoxin. J. Biol. Chem. **246**:3953-3960.
128. Tanaka, M., T. Nakashima, A. Benson, H. Mower, and K. T. Yasunobu. 1966. The amino acid sequence of *Clostridium pasteurianum* ferredoxin. Biochemistry **5**:1666-1681.
129. Thomas, D. A., and H. K. Kuramitsu. 1971. Biosynthetic L-theronine deaminase from *Bacillus stearothermophilus*. I. Catalytic and regulatory properties. Arch. Biochem. Biophys. **145**:96-104.
130. Thompson, P. J., and T. L. Thompson. 1962. Some characteristics of a purified heat-stable aldolase. J. Bacteriol. **84**:694-700.
131. Tsunoda, J. N., K. T. Yasunobu, and H. R. Whiteley. 1968. Non-heme iron proteins. IX. The amino acid sequence of ferredoxin from *Micrococcus aerogenes*. J. Biol. Chem. **243**:6262-6272.
132. Ulrich, J. T., G. A. McFeters, and K. L. Temple. 1972. Induction and characterization of β-galactosidase in an extreme thermophile. J. Bacteriol. **110**:691-698.
133. Waugh, W. F. 1954. Protein-protein interactions. Advan. Protein Chem. **9**:326-437.
134. Weerkamp, A., and R. D. MacElroy. 1972. Lactate dehydrogenase from an extremely thermophilic bacillus. Arch. Microbiol. **85**:113-122.
135. Welker, N. E., and L. L. Campbell. 1963. Induced biosynthesis of α-amylase by growing cultures of *Bacillus stearothermophilus*. J. Bacteriol. **86**:1196-1201.
136. Welker, N. E., and L. L. Campbell. 1963. De novo synthesis of α-amylase by *Bacillus stearothermophilus*. J. Bacteriol. **86**:1202-1210.
137. Wisdom, C., and N. E. Welker. 1973. Membranes of *Bacillus stearothermophilus:* factors affecting protoplast stability and thermostability of alkaline phosphatase and reduced nicotinamide adenine dinucleotide oxidase. J. Bacteriol. **114**:1336-1345.
138. Yarbrough, L. R., and H. Koffler. 1972. The nature of a relatively heat-stable flagellin from thermophilic cells. Conf. Extreme Environ., Ames Research Center, NASA, Moffett Field, Calif., 26-28 June 1972.
139. Yasunobu, K. T., and J. McConn. 1970. *Bacillus subtilis* neutral protease, p. 569-575. *In* G. E. Perlman and L. Lorand (ed.), Methods in enzymology, vol. 19. Academic Press Inc., New York.
140. Yoshida, M. 1972. Allosteric nature of thermostable phosphofructokinase from an extreme thermophilic bacterium. Biochemistry **11**:1087-1093.
141. Yoshida, M., and T. Oshima. 1971. The thermostable allosteric nature of fructose-1, 6-diphosphatase from an extreme thermophile. Biochem. Biophys. Res. Commun. **45**:495-500.
142. Yoshida, M., T. Oshima, and K. Imahori. 1971. The thermostable allosteric enzyme: phosphofructokinase from an extreme thermophile. Biochem. Biophys. Res. Commun. **43**:36-39.
143. Yoshizaki, F., T. Oshima, and K. Imahori. 1971. Studies on phosphoglucomutase from an extreme thermophile, *Flavobacterium thermophilum HB8*. I. Thermostability and other enzymatic properties. J. Biochem. (Tokyo) **69**:1083-1089.
144. Zeikus, J. G., and T. D. Brock. 1971. Protein synthesis at high temperatures: aminoacylation of t-RNA. Biochim. Biophys. Acta **228**:736-745.
145. Zeikus, J. G., and T. D. Brock. 1972. Effects of thermal addition from the Yellowstone geyser basin on the bacteriology of the Firehole river. Ecology **53**:283-290.